Faculty of Resource Science and Technology

GENOTYPING *ESCHERICHIA COLI* IN ANIMAL FAECES USING PULSED FIELD GEL ELECTROPHORESIS (PFGE)

Sarina anak Niyup

Bachelor Science with Honours

(Resource Biotechnology)

2008

GENOTYPING *ESCHERICHIA COLI* IN ANIMAL FAECES USING PULSED FIELD GEL ELECTROPHORESIS (PFGE)

Sarina anak Niyup

This project is submitted in partial fulfillment of the requirements for the degree of

Bachelor Science with Honours

(Resource Biotechnology)

Department of Molecular Biology

Faculty Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2008

ACKNOWLEDGEMENT

To God, for pouring all His blessing by giving me strength and wisdom in completing this project. I would like to thank my family for supporting me all the time and to my supervisor, Assoc. Prof. Dr. Kasing Apun, for her guidance and help towards me throughout my final year project.

Special gratitude to Miss Chong Yee Ling for her advice and assistance in this project, Encik Azis and Encik Amin for providing me technical assistance for me whenever I need. Thanks also to Mr Chang, who helped me in my swine sampling process.

I would like to extend my appreciation to the owner of chicken rearing-farm (Sungai Moyan chicken rearing-farm) and swine farm (Bau-Musi swine farm) for giving chance to me to do my sampling. To all post-graduates students, for their valuable advices, concerns, and supports to improve my laboratory skills.

Finally, to my fellow partners in Microbiology Lab, Miss Leanne Marian Hong and Miss Delima anak Andrew Buntak, as well as, all my coursemates for their companionship and moral support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

cfu	colony forming units
DNA	deoxyribonucleotide acid
EDTA	ethylenediamine tetra-acetic acid
LB	Luria Bertani
M	Mole
mL	milliliter
mM	millimole
NaCI	Natrium chloride
PFGE	Pulsed Field Gel Electrophoresis
rpm	revolution per minute
sdH_2O	sterile distilled water
SDS	Sodium Dodecyl Sulfate
TBE	Tris Boric EDTA electrophoresis buffer
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) methylamine
UV	Ultravoilet
V	Volts
mm	micrometer
µL	microlitre
%	percentage
^oC	degree celcius

Genotyping *Escherichia coli* in animal faeces using Pulsed-field Gel Electrophoresis (PFGE)

Sarina anak Niyup

Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Escherichia coli is one of a group of bacteria that is predominantly present in the intestines and faeces of warm-blooded mammals, including wildlife, livestock, and humans. Pulsed field gel electrophoresis had been applied widely to study the genetic diversity of *E. coli*. In this study, genetic variations of 25 *E. coli* isolates from different animals; cattle, chicken, and swine, as commercial livestock was examined by pulsed field gel electrophoresis digested with the restriction enzyme, *Xba1*. PFGE result that was analyzed using Dice Correlation and UPGMA methods revealed the highest similarities of *E. coli* between cattle and chicken, which is 64.38%. The highest similarities of *E. coli* within animals, was found within chicken faeces, which is 70.37%. PFGE results successfully categorised *E. coli* to their sources and showed a close genetic relationship (53.64% to 70.37%) between *E. coli* strains belonging to the same animals. The result obtained showed that PFGE is a powerful tool to explore the genetic relationship among *E. coli* isolates in animal faeces.

Keywords: *Escherichia coli*, genetic relationship, *Xba1*, PFGE.

ABSTRAK

Escherichia coli ialah kumpulan bakteria yang terbesar dalam saluran pemakanan dan dalam tahi mamalia berdarah panas termasuk hidupan liar, haiwan ternakan, dan manusia. PFGE telah digunakan untuk mengkaji kepelbagaian genetik E. coli. Dalam kajian ini, kepelbagaian genetik daripada 25 pencilan E. coli antara haiwan-haiwan terutamanya lembu, ayam, dan babi yang menjadi haiwan ternakan bagi sumber makanan manusia telah diuji menggunakan PFGE setelah dihadam dengan enxim sekatan Xba1. Keputusan PFGE yang telah dianalisis menggunakan cara Dice Correlation dan UPGMA menunjukkan kesamaan tertinggi di antara E. coli di antara lembu dan ayam dengan 64.38%. Kesamaan tertinggi E. coli dalam ketiga-tiga haiwan tersebut dijumpai dalam ayam dengan 70.37%. PFGE telah berjaya mengkategorikan E. coli kepada sumber masing-masing dan menunjukkan hubung kait genetic yang jelas (53.64% hingga 70.37%) di antara E. coli dari haiwan yang sama. Keputusan yang diperolehi telah membuktikan PFGE berkebolehan untuk mengkaji hubung kait antara pencilan E. coli dalam tahi haiwan.

Kata kunci: Escherichia coli, hubung kait genetik, Xba1, PFGE.

1.0 INTRODUCTION

Escherichia coli is one a group of bacteria that normally prominent in the intestines and faeces of warm-blooded mammals, including wildlife, livestock, and humans (Meays *et al*. 2004). Livestock such as cattle, chicken, and swine are the major provider to human daily food consumption. Transmission of this pathogen to the water and food supply mainly from livestock has indirectly linked animals as they act as reservoirs (Pell, 1997). Bacterial pathogenic, *E. coli* O157:H7 cause human threat because it is associated with the ability to produce Shiga-toxins (*Stx*). Transmission from cattle, chicken, and swine to human has caused potential human threat to human health. However, it is difficult to clarify whether shiga-toxin 2-producing strains may represent a cause of avian disease or even a possible health hazard for humans (Caprioli *et al*. 2004). The strain was not only isolated from cattle but also from other animals such as birds or flies as well as from feeds and drinking water.

E. coli population is essentially clone in nature and experienced infrequent recombination events (McLellan *et al*. 2003). Thus, *E. coli* genotype in each animal will be different within each animal as different animal has their own characteristics that will support growth of this bacterium in their gastrointestinal tract (Wang *et al*. 1996). Collections of *E. coli* isolated from gastrointestinal tract are genetically distinct from collections isolated from environmental conditions outside the host selected for specific clonal attributes (Topp *et al*. 2003). In this study, it is found that *E. coli* community in the swine gut is variable from individual and changes occur during lifetime of the animals. However, degree to which host influences the genetic structure of *E. coli* still remains in question (McLellan *et al*. 2003).

The analysis of *E. coli* in term of genotype will be done using Pulsed Field Gel Electrophoresis (PFGE). PFGE gave a higher identification of closely related strains than repetitive extragenic palindromic (REP) PCR fingerprinting and enterobacterial repetitive intergenic consensus PCR since PFGE can detect single base pair changes which reported by McLellan *et al*. (2003). As reported by Bopp *et al*. (2002), PFGE was very useful in sub-typing *E. coli* as it was reproducible and has sufficient discriminatory power to allow detection of minor genetics variation amongst isolates. In addition, future investigations using PFGE may tolerate the detection of human disease associated clones, explanation of their particular epidemiologies for development of advance methods to exclude the pathogenic bacteria from the carcasses before being process (Duffy *et al*. 2005).

Faeces samples taken from each farm; cattle, chicken, and swine respectively will be analyses for occurrence of *E. coli* by determine the sample concentrations in cfu/ml. While, genetic relationship between cattle, chicken, and swine will be analyzed using RAPDistance 1.04 package analysis which is available on the internet based on Dice correlation matrix. Dendogram will be constructed in MEGA 4.0 format based on unweighted pair group method (UPGMA) to describe the genetic relations between *E. coli* from different animal host.

The objectives of this project are to:-

i. determine the occurrence of *Escherichia coli* in different faeces respectively from cattle, chicken, and swine.

ii. determine degree of variation of *Escherichia coli* from cattle, chicken, and swine using PFGE.

2.0 LITERATURE REVIEW

2.1 *Escherichia coli*

The Gram-negative bacterium *Escherichia coli* is a major member of the bacterial microbiota of the environment and in the faeces of species of birds and mammals such as chicken, swine and cattle (Parveen *et al*. 2000; Higgins *et al*. 2007). *E. coli* colonizes the human gastrointestinal tract and warm-blooded animal (Parveen *et al*. 2000).

E. coli is classified into five main categories known as Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC) (or Verotoxigenic *E. coli* (VTEC)), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC) (or Shiga toxin-producing *E. coli*), and Enteroaggregative *E. coli* (EAEC) (Duffy *et al*. 2004). Among all, *E. coli* O157:H7 which is one of a member of enterohemorrhagic *E. coli* (EHEC) group I, contain common sets of virulence genes that produce Shiga toxins (*stx*1 and *stx*2 also known as VT1 and VT2), attachment effacement (*eae*) and enterohemolysin genes (*hlyA*) (Hahm *et al*. 2003) is commonly employed indicator faecal pollution of water, and this bacterium can also possess virulence factors enabling it to cause, in the case of the O157:H7 serotype, fatal disease.

Enterotoxigenic *E. coli* (ETEC) and Shiga-toxin producing *E. coli* (STEC), which also called verotoxin-producing *E. coli* (VTEC), have causes postweaning diarrhea (PWD). This disease is usually the main infectious problem of large-scale farms and cause for significant losses worldwide (Khac *et al*. 2006; Osek, 2000). Porcine pathogenic *E. coli* involved in PWD typically belong to serogroups O8, O138, O139, O141, O149, and O157. Serogroup O149 seems to be predominant serogroup in most countries (Khac *et al*. 2006). ETEC and STEC

implicated in PWD possessed F4 (K88) and F18 fimbrial adhesions as well as LTI, STI, STII enterotoxin and shiga toxin virulence markers (Osek, 2000).

Jimenez *et al*. (2003) found in both types of samples (broiler chicken carcasses with and without visible faecal contamination), *E. coli* was most frequently identified bacteria compare to *Enterobacter cloacae*, despite both are being predominant species isolated in all samples. Along this study, it is found that *E. coli* is more susceptible to chlorination than *Ent. cloacae* during prechilled process along poultry processing plant. However, *E. coli* O157:H7 isolated from poultry is rarely reported because there is no direct link with the consumption of poultry. The O157 disease in humans has been found, but an O157 food-borne outbreak was reported where poultry was on the menu. Study done by Best *et al*. (2003) suggest that Shiga-toxins have little or no involvement in the persistent colonization of chicken and found that poultry could be a major risk to human health.

Domestic cattle are regarded as the main reservoir of human pathogenic STEC (Urdahl *et al*. 2003; Duffy *et al*. 2004). Many outbreaks linked to beef and beef products and STEC is quite prevalent in cattle and there are 70% and 100% of cattle *stx* positive. Consumption of undercooked beef and unpasteurized milk products associated in foodborne illness due to *E. coli* O157:H7 (Berry *et al*. 2005). Serotypes O22:H8, O26:H11, O91:H21, O113:H4/H21 and OX3:H2/H21 is among most frequently isolated from cattle (Urdahl *et al*. 2003).

2.2 *Escherichia coli* isolated from animal faeces

Slurry is consists major of 90-95% of water, faeces, urine and feed particles. Microbes from manure are often low on the priority list of control and remediation, despite the fact that several outbreaks of gastroenteritis have been traced to livestock operation (Pell, 1997). In 1990s, it was one of the challenges that farmers face in maintaining profitability in dairy industry as well as protecting water quality and the health of humans and animals. However, in 2003, the world especially from livestock production region has been intensified in proximity to grow in human populations. Thus, increasing public and regulatory concern about the protections of water resources from contamination was done by runoff from land that is fertilized with livestock waste (Topp *et al.* 2003). In addition, faecal pollution degrades water quality; thus, restrict its use for harvesting seafoods and recreational activities (Parveen *et al*. 2000).

The study performed by Aitken *et al*. (2007) found a non-pathogenic strain of *E. coli* was isolated from a sample of manure. *E. coli* is identified at 50% when coming from broiler carcasses that were not visibly contaminated with faeces and at 59.7% when came from the visibly contaminated broiler carcasses (Jimenez *et al*. 2003). Study by Salter *et al*. (1998) reported *E. coli* O157:H7 was found to grow in the range 44-45.5ºC just as the faecal coliform group does. Berry *et al*. (2005) found horizontal transmission by direct or indirect faecal-oral exposure appears to be the primary means of dissemination of *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) in cattle. While, Duffy *et al*. (2005) agreed that VTEC are frequently shed in the faeces of healthy animals and contaminating the bovine hide.

2.3 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) one of the current methods of DNA fingerprinting with rare-cutting restriction enzymes coupled with electrophoretic analysis and have an ability to discriminate species (Meays *et al.* 2004). Wang *et al.* (1996) reported on DNA fingerprinting by pulsed field gel electrophoresis was used to differentiate *E. coli* O157:H7 strains in order to understand the survival and growth characteristics in faeces of strain variation.

According to Basim & Basim. (2000) state that PFGE is a technique with concept that DNA molecular larger than 50kb can be separated by using two alternating electric field and this principle has been developed, where pulsed fields demonstrated for separating DNAs from a few kb to over 10 megabase pairs (Mb). There are seven types of instruments have been utilized in pulsed electrophoresis which are Field-Inversion Gel Electrophoresis (FIGE), Transverse-Alternating Field Gel Electrophoresis (TAFE), Contour-Clamped Homogeneous Electric Field (CHEF), Orthogonal-Field Alternation Gel Electrophoresis (OFAGE), Rotating Gel Electrophoresis (RGE), Programmable Autonomously-Controlled Electrodes (PAGE), and Pulsed-Homogenous Orthogonal Field Gel Electrophoresis (PHOGE). Among all, CHEF is widely used apparatus that provides a more sophisticated solution to the distorting effect of both the edges of the chamber and the passive electrodes. CHEF has 24 points electrodes and sets of voltages are equally spaced around the hexagonal contour. There are no "passive" electrodes because all electrodes are connected to the power supply by the external loop of resistors. So, CHEF can produce sufficient power supply uniformly to produce straight lanes on the run gel. The angle of orientation is 120º with radiating electro-potential gradiations from positive to the negative poles. Up to 7000kb molecules can be separated by CHEF.

Applications of PFGE increased with the development of two orders of magnitude the size of DNA molecules that can be routinely fractionated and analyzed. PFGE has shown excellent ability to fraction small, natural linear chromosome DNAs ranging in size from 50kb to multimillion-bp varies from bacteria and viruses to mammals. The new technique in PFGE is the ease with which the genome size can be measured, which previously considered error if measured by other techniques. PFGE can be used to construct physical map with the use of restriction endonuclease digestion. It has proven that PFGE is extremely powerful to analyze large DNA molecules including specifically fragmented genomes of bacteria, mammals, parasite protozoa, and intact chromosomal DNAs from fungi (Basim & Basim. 2000).

PFGE is also useful for sub-typing *E. coli* O157:H7 isolates during outbreak investigations because PFGE is reproducible and has sufficient discriminatory capability to allow detection of minor genetic variations among isolates (Limberger *et al.* 2002). PFGE typing has been frequently used to mark out sources of outbreaks and in tracking the models of movement of clones from animals, to food and human cases of verotoxigenic infection (Duffy *et al*., 2004). Khac *et al*. (2006) and Osek, (2000) agreed that PFGE is a powerful tool to reveal inter- and intra-serotype specific genetic differences among porcine pathogenic from pigs suffering from post-weaning disease (PWD).

In fact, PFGE gave a higher identification of closely related strains than repetitive extragenic palindromic (REP) PCR fingerprinting and enterobacterial repetitive intergenic consensus PCR (McLellan *et al.* 2003). Due to the ability, PFGE has been chosen in this study. Even though *E. coli* populations does not experience frequent recombination events, natural clonal is essential as host-specific environments may account for part of the observed

diversity within *E. coli* population. As reported by Meays *et al*. (2004), PFGE are extremely sensitive to minute genetic differences.

3.0 MATERIALS AND METHODS

3.1 Sampling sites and collection

Duplicates of ten samples of fresh faeces from chicken and duplicates of fifteen swine respectively were collected from identified farms located around Kuching and Bau-Musi. Faeces collections were done from early November 2007 until end of December 2007. Approximately, small amount of fresh faeces was collected from the ground and divide into two stomacher bags. Then, the samples put in sterile ziplock bag or container (Lynn *et al*. 1997) and kept cold at 4°C with ice bags during transportation (Wang *et al*. 1996). Analysis started within 3 hours after recovery from animals (Wang *et al*. 1996) or stored in cold room for a long storage. *E. coli* isolates from Empilia Village cattle faeces glycerol stock was recovered in LB broth and incubated with shaking at 37ºC for 16 hours before undergo DNA harvesting protocol for Pulsed Field Gel Electrophoresis (PFGE).

3.2 Identification of *Escherichia coli* in animal's faeces

About 2.5 grams of fresh faeces from each sample was enriched with 22.5 ml of Tryptone Soya Broth (TSB) (Oxoid, England) supplemented with 20 mg/L novobiocin (Oxoid, England) (Dontorou *et al*. 2004). All faecal samples were serially diluted and spread plated onto MacConkey agar (Oxoid, England) for total coliform count. Then, samples are incubated at 37°C for 16 hours. After the incubation process, faecal samples were serially diluted in Buffered Peptone Water (BPW) (Oxoid, England) and spread plated onto cefixime-tellurite Sorbitol MacConkey agar, CT-SMAC agar (Oxoid, England) and incubate for another 24 hours. Two colonies selected from CT-SMAC agar were streaked on Eosin Methlyene Blue (EMB) (Levine, Oxoid, England) by using a loop for bacterial isolation. The next day,

bacterial colonies that showed green-metallic sheen colouration were subcultured onto nutrient agar as stock culture and remaining isolates were undergo a series of biochemical tests in order to identify the identity of bacteria isolated. These standard tests include Gram-staining, Simmon citrate utilization test, methyl red test, Voges-Proskauer test, indole test, SIM test and TSI test.

Bacterial smears were prepared before Gram-staining. Culture from nutrient agar was transferred using sterile inoculating loop and emulsified with a drop of water on the center of the slide. Heat fixation was performed by the rapid passage of the air-dried smear two or three times over the flame of the Bunsen burner. Then, differential staining was performed by staining with crystal violet for 20 minutes. Gentle wash was applied to the stain with tap water for 2 seconds. Next, Gram's Iodine was applied for one minute before decolourized with 95% alcohol for 10-20 seconds until all solvent flows colourlessly. After that, the stain was washed with tap water for two seconds. Lastly, counterstain with safranin for 20 seconds and washed with tap water for 2 seconds before blot dried with bibulous paper. Dry Gram-stain was observed under microscope with pink colour stain for Gram-negative bacteria and violet colour stained for Gram-positive.

While, Simmon citrate utilization test was done by inoculating a colony of bacteria using sterile inoculating needle into Simmon citrate agar by means of streak inoculation and incubated for 24 to 48 hours at 37ºC. Methyl red test was performed by inoculating colony of bacteria using sterile loop into MR-VP broth and incubated at 37ºC for 24 to 48 hours. Voges-Proskauer test performed by adding Barritt's reagent into MR-VP broth and positive reactions occur at once or within five minutes.

Indole test was performed by inoculating a colony of bacteria using sterile inoculating needle into SIM medium and incubated for 24 to 48 hours. TSI (Triple Sugar-Iron) test was performed on triple sugar-iron agar slant by inoculating colony of bacteria into triple sugar-iron agar by means of stab-and-streak inoculation and incubated for 18 to 24 hours.

Further identification was done to identify *E. coli* strain examined using API® 20E kit test (BioMerieux, France). First, single well-isolated fresh colony from isolation plate was removed and carefully emulsified in 5 ml of NaCI 0.85% to achieve a homogenous bacterial suspension. API® 20E incubation box was filled with 5 ml of sterile distilled water. Then, both tube and cupule test CIT, VP, and GEL were filled with bacterial suspension while other test only filled up the tube and not the cupule. Mineral oil overlaid on test ADH, LDC, ODC, H_2S, and URE to create anaerobic condition. After that, the incubation box closed and incubated at 36ºC ± 2ºC for 18-24 hours. After incubation period, the strip was read by referring to the Reading Table. All spontaneous reactions were recorded on the result sheet if 3 or more tests (GLU test + or -) are positive, and revealed the tests which require the addition of reagents, which are TDA test, IND test, and VP test. If the number of positive tests (including the GLU test) before adding of reagents is less than 3, the strip have to reincubate for further 24 hours without reagent added and revealed the test required for addition of reagents.

3.3 Pulsed Field Gel Electrophoresis Plug Preparation

Pulsed Field Gel Electrophoresis plug was prepared based on methods by Apun *et al*. (2005) with slight modification. Single colony from Nutrient Agar (Oxoid, England) including wild strain of *E. coli* (EDL 933) as positive control isolate was recovered from glycerol stock were inoculated into 10 ml of Luria Bertani Broth (LB) (Miller, Himedia Lab) and incubated for 16 hours with gentle agitation at 200 rpm using incubator shaker (Innova^{TM}, 4000). Bacteria recovered from glycerol stock should be sub-cultured once or twice before using the specimen.

On the next day, 700 µl of bacterial suspension were transferred into autoclaved micro-tube and harvested by centrifugation using microcentifuge (Mikro 22R, Hettich Zentrifugen) at 8000 rpm for five minutes at 4ºC. Then, the cell pellet obtained was washed with 1.0 mL of cold SB (10 mM Tris [pH 7.5], 1 M NaCI). The suspension was centrifuged again at 8000 rpm for five minutes at 4ºC and supernatant was discarded. The cell pellet was resuspended in 0.5 mL of warm SB and placed in the water bath shaker (Labtech, Daihan) at 52ºC. Then, equal volume of molten 1.8% LMP agarose (Ultra Pure, GIBCOBRL USA) diluted in LMP buffer (10 mM Tris [pH 7.5], 100 mM Na-EDTA [pH 8.0], and 20 mM NaCI) equilibrated at the same temperature was added into the cell suspension. The cell suspension was mixed with molten 1.8% LMP agarose by inverting the tube slowly up and down. Later, the each mixture was carefully pipetted into two plug molds (BioRad Laboratories) and allowed to harden at 4ºC for 15 minutes. After the agarose plugs solidified, the plug was transferred into autoclaved universal bottle contained 3 mL of lysis solution (10 mM Tris [pH 7.5], 50 mM NaCI, 100 mM Na-EDTA, 0.5 % Sarcosyl, and 1 mg/mL lysozyme). The DNA plugs were incubated in water bath shaker at 37ºC for 6 hours. Then, lysis solution was

removed from the universal bottle that contained DNA plugs and change with 3 mL of ES solution (0.5 mM Na-EDTA [pH 8.0], 1% Sarcosyl, and 1 mg/mL proteinase K). The plugs were incubated for 16 hours in water bath shaker at 50ºC.

After that, the plugs were washed with sterile TE buffer (10 mM Tris-base [pH 7.5], and 1 mM Na-EDTA [pH 8.0]) in water bath at 37ºC with gentle agitation for two times at one hour interval and subsequently for three times at two hours intervals. The washed DNA plugs were stored in sterile TE buffer for two weeks storage or in 0.5 M Na-EDTA for long storage at 4ºC or can be subjected to restriction endonuclease (RE) digestion instantly.

3.4 Restriction Endonuclease Digestion

DNA plugs were cut into size of 1 mm × 1 mm × 5 mm using alcohol- flamed scalpel blade and transferred into autoclaved microcentrifuge tube contained 200 µL of sterile TE. The plugs were incubated on ice for 30 minutes with the tube being inverted for several times). TE was removed and fresh TE was added for further 30 minutes.

Next, the plugs were chilled on ice with 50 µL of 1 × restriction endonuclease buffer and 0.1 mg/mL BSA (Promega) for further 30 minutes. RE buffer mix was removed and replaced with 50 µL of fresh mixture of 1 × RE buffer, 0.1 mg/mL BSA, and 20 units of *Xba1*. The plugs were placed on ice for another 15 minutes before incubated for 16 hours at 37ºC with gentle shaking.

3.5 Pulsed Field Gel Electrophoresis Run

After the digestion, the slice was equilibrated with 200 µL 0.5 × TBE buffer (45 mM Tris-Base, 45 mM boric acid, 1 mM Na-EDTA [pH 8.0]) on ice for 45 minutes. The slice was loaded into the well of 1.2% Ultra Pure Pulsed Field Certified Agarose (Bio-Rad Laboratories) prepared in 0.5 × TBE buffer. The wells were sealed with the remainder of the molten 1.2% agarose gel. Then, the gel was pre-chilled at 4ºC for 5 minutes.

The gel performed by using contour-clamped homogenous electric field on a CHEF-DR III (Bio-Rad Laboratories) with two liters of standard 0.5 × TBE buffer. PFGE was run at 6V/cm and reorientation angle at 120º with temperature at 14ºC with two blocks of pulse time ramped from 15.0 seconds to 30.0 seconds for 9 hours and 2.2 seconds to 56.0 seconds for 17 hours, respectively. DNA size standard that was used was PFG lambda ladder (Biolabs, New England) consists of concatemers ranging from 48.5 to 1000kbp. The gel was stained in 0.5 µg/mL ethidium bromide for 20 seconds and destained in distilled water for 15 minutes.

Electrophoretic patterns were visualized under UV light transilluminator (Ultra-Lum, California) and recorded into computer system (Wang *et al*. 1996 and Pang *et al*. 2007).